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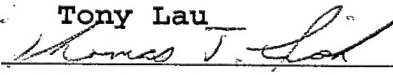
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## Introduction

It has long been thought that environmental factors contribute to the neurodegeneration characteristic of many neurological diseases. In the past decade, considerable attention has been directed toward environmental agents that inhibit mitochondrial function. This work has provided evidence that mitochondria provide signals to cells, including neurons, that initiate activation of processes leading to programmed or "apoptotic" cell death. The goals of this contract are: 1) to first characterize toxin-induced alterations of respiratory chain redox status and mitochondrial membrane potential in intact functioning neuronal populations; 2) to examine the role of specific intracellular mediators of mitochondrial and neuronal dysfunction including release of mitochondrial cytochrome c, calcium overload, reactive oxygen species (ROS), and mitochondrial permeability transition. Experiments were to be conducted in intact functioning brain slices preparations using optical methodology to monitor changes in mitochondrial function, and electrophysiological methods to monitor neuronal function.

## Body

In the first year of this contract, experiments were conducted to establish baseline responses of mitochondrial redox activity and hippocampal electrophysiology to toxin exposure. We also established dose response relationships for the putative Parkinson's disease toxin, 1-methyl-4-phenylpyridium (MPP<sup>+</sup>), the excitotoxin N-methyl-D-aspartate (NMDA), and 3-nitropropionic acid (3-NP), a mitochondrial toxin used to model Huntington's disease. In the second year of the contract we established an organotypic slice culture model to directly assess the effects of toxin exposure on neuronal cell death, and we began studies of rotenone, a mitochondrial Complex I inhibitor that had been shown to induced Parkinson's-like neuropathology in experimental animals. In this report, we present data that concludes some of these earlier studies. We also show new data describing the effects of the toxins on mitochondrial ROS production in hippocampal slices. Finally, we address the hypothesis that mitochondrial permeability transition plays a role in toxin-induced neuronal damage.

### Delayed Hippocampal Cell Death After Toxin Exposure

In these experiments, organotypic hippocampal slice cultures were grown for 2-3 weeks in culture and then exposed for 1 hr to toxins. Cell death was examined 24 hr, 48 hr and 72 hr later using the fluorescent dye propidium iodide, that only stains cells with damaged plasma and nuclear membranes. In our previous report, we provided preliminary data that mitochondrial Complex I inhibitors could cause selective neuronal cell death in hippocampal subfield CA1 compared to subfield CA3. In this respect, it appeared that inhibition of Complex I with mitochondrial toxins produced damage similar to that observed in hypoxia/ischemia. Such damage had not been reported earlier in studies that examined damage to the striatum and substantia nigra following exposure of experimental animals to these toxins. In this report, we present the conclusion of this study in manuscript form (Appendix). We found that the respiratory chain Complex I inhibitor rotenone, and to a lesser extent MPP<sup>+</sup>, selectively damaged neurons in

hippocampal subfield CA1. The Complex II inhibitor, 3-NP, produced little neurotoxicity, and the excitotoxin NMDA caused damage to both the CA1 and CA3 subfields.

#### Mitochondrial Permeability Transition After Toxin Exposure

Mitochondrial permeability transition (MPT) is characterized by a sudden increase in permeability of the inner mitochondrial membrane, collapse of the mitochondrial membrane potential, and release of pro-apoptotic signaling molecules such as cytochrome c. MPT has been shown to occur under a variety of conditions although mainly in cell culture and in isolated mitochondrial preparations. There have been a few reports that MPT may occur after ischemia in the intact brain but these studies have not been conclusive. A major goal of this contract was to determine if there is evidence of MPT following toxin exposure.

**Mitochondrial Membrane Potential** – A key feature of MPT is collapse of the mitochondrial membrane potential. We measured changes in mitochondrial membrane potential in hippocampal slices using the fluorescent indicator JC-1. JC-1 accumulates in mitochondria in proportion to the mitochondrial membrane potential. At high concentrations the dye shows red fluorescence while in low concentrations its fluorescence is green. Thus, the ratio of red/green fluorescence is indicative of the mitochondrial membrane potential. Hippocampal slices were exposed to either  $\text{MPP}^+$ , 3-NP, NMDA, or rotenone. In these experiments, we used relatively high doses of each toxin. These toxin doses had been previously shown to produce significant cell death (with the exception of 3-NP) in organotypic slice cultures (see above). Summaries of the data are shown in Figures 1 and 2. Figure 1 shows the 1 hr time course of changes in the JC-1 fluorescence ratio following toxin exposure. Figure 2 shows a comparison of the groups 60 min after exposure. Only the Complex I inhibitors  $\text{MPP}^+$  and rotenone caused significant mitochondrial depolarization 60 min after exposure. NMDA, which has been reported to induce MPT in neuronal cell cultures, had no effect of mitochondrial membrane potential in hippocampal slices. It should also be noted that the changes in mitochondrial membrane potential were much smaller than FCCP, a toxin that completely collapses the mitochondrial membrane potential.

At the doses used, all of the toxins produced complete and rapid inhibition of synaptic transmission in hippocampal slices, and except for 3-np, produce significant cell death in slice cultures. Thus, the data do not support the hypothesis that MPT occurs following toxin exposure. However, it remains possible that the small mitochondrial depolarization observed after exposure to Complex I inhibitors might reflect MPT in a small population of cells, since the method averages changes from a relatively large volume of tissue. It will be necessary to examine changes in mitochondrial membrane potential in single cells following toxin exposure. In future studies, we will use confocal microscopy to detect changes in mitochondrial membrane potential in single cells in organotypic slice cultures, where cell death can also be examined.

Other Indicators of MPT in Brain Slices – Because the mitochondrial toxins did not provide clear evidence of MPT in brain slices, we examined changes in mitochondrial membrane potential under conditions that have been shown to induce MPT in neuronal cell culture preparations. We examined four conditions: exposure to MPT inducing agents tert-butyl-hydroperoxide (t-BuOOH), thapsigargin (TG), zinc – high potassium, and oxygen-glucose deprivation (OGD). TG and t-BuOOH, at doses shown to cause MPT in neuronal cultures, had no effect on mitochondrial membrane potential in hippocampal slices. Zn-K<sup>+</sup>, and to a greater extent OGD, caused mitochondrial depolarization in hippocampal slices. However, the change in mitochondrial membrane potential was not inhibited by Cyclosporin A, a signature inhibitor of MPT in a variety of cell types and in isolated mitochondria. These data are shown in Figures 3 and 4. We must tentatively conclude that MPT does not readily occur in hippocampal brain slices. It is possible that cells in intact brain acquired from adult animals are relatively resistant to MPT in comparison to isolated mitochondria and cultured cells acquired from relatively young animals.

#### Mitochondrial Reactive Oxygen Species (ROS) Production and Toxin Exposure

Mitochondria are thought to be an important source of free radical production in a variety of neurological conditions. To examine ROS production after exposure to MPP<sup>+</sup>, rotenone, 3-NP, or NMDA, we used the fluorescent indicator dihydrorhodamine 123. Non-fluorescent dihydrorhodamine 123 is oxidized to fluorescent rhodamine 123 by H<sub>2</sub>O<sub>2</sub>. Hippocampal slices were loaded with dihydrorhodamine 123 and exposed to doses of MPP<sup>+</sup>, rotenone, 3-NP, or NMDA that previously caused cell death in organotypic slice cultures. A summary of the results is shown in Figure 5. All toxins caused an increase in dihydrorhodamine 123 fluorescence. Statistical comparisons are provided in Figure 6 for data taken 60 min after toxin exposure. By 60 min, all toxins showed a significant increase in dihydrorhodamine 123 fluorescence compared to control.

These data indicate that ROS rproduction increases after exposure to Complex I inhibition, Complex II inhibition, or excitotoxicity. The data suggest that conditions would be favorable for MPT since mitochondrial oxidation promotes MPT in a variety of cell types and in isolated mitochondria. However, we found no indication of MPT in hippocampal slices, as described above.

#### **Key Research Accomplishments**

- 1) The Complex I inhibitors, MPP<sup>+</sup> and rotenone cause selective damage to pyramidal cells in hippocampal subfield CA1. This pattern of cell damage is similar to that produced by hypoxia/ischemia.
- 2) Only Complex I inhibition caused significant depolarization of mitochondrial membrane potential in hippocampal slices.
- 3) We have been unable to produce evidence of mitochondrial permeability transition in hippocampal slices.
- 4) All toxins tested produced increased dihydrorhodamine 123 fluorescence indicating increased ROS production.

## Reportable Outcomes

Xu, G.-P, Perez-Pinzon, M.A., and Sick, T.J. Mitochondrial Complex I Inhibition Produces Selective Damage to Hippocampal Subfield CA1, Neurotoxicity Research, Submitted for Publication.

## Conclusions

The results documented here indicate that inhibitors of mitochondrial Complex I kill pyramidal cells in the hippocampus and that damage is selective for the CA1 subfield. Complex I inhibitors also cause mitochondrial depolarization and production of reactive oxygen species in subfield CA1. However, although mitochondrial depolarization and ROS production might signal mitochondrial permeability transition (MPT), we have, as yet, no direct evidence that MPT occurs in hippocampus. This is an important point because MPT has been a major focus of research in the field of neurodegeneration.

## Appendix

Xu, G.-P., Perez-Pinzon, M.A., and Sick, T.J. Mitochondrial Complex I Inhibition Produces Selective Damage to Hippocampal Subfield CA1, Neurotoxicity Research, Submitted for Publication.

## Figure Legends

Figure 1. Effects of toxin exposure on mitochondrial membrane potential (JC-1 fluorescence) in hippocampal subfield CA1. Slices were incubated in JC-1 and then exposed to ACSF (Control), 1 mM MPP<sup>+</sup>, 1 mM 3-NP, 100  $\mu$ M NMDA, or 10  $\mu$ M rotenone. Values are means  $\pm$  SEM, n = 6.

Figure 2. Comparison of relative mitochondrial membrane potential values (normalized JC-1 fluorescence ratios) 60 min after exposure to toxins (doses same as in Fig. 1). \*\* p < .01 compared with control.

Figure 3. Examples of the changes in mitochondrial membrane potential after exposure of hippocampal slices to 10  $\mu$ M thapsigargin or 150  $\mu$ M tert-butylhydroperoxide (t-BuOOH). These agents have been shown to promote mitochondrial permeability transition (MPT) in isolated mitochondria and cultured neurons.

Figure 4. Relative changes in mitochondrial membrane potential following exposure of hippocampal slices to 300 nM Zn<sup>+</sup> - 60 mM K<sup>+</sup> (top graph) or oxygen-glucose deprivation (lower graph) sufficient to cause 20 min of cellular depolarization (AD-anoxic depolarization). While both Zn-K and oxygen-glucose deprivation caused mitochondrial depolarization, the depolarization was not inhibited by Cyclosporin A (CsA).



Figure 5. Time course of changes in dihydorhodamine 123 fluorescence following exposure of hippocampal slices to the indicated toxins. Toxin doses were the same as those shown in Figure 1.

Figure 6. Comparison of normalized dihydorhodamine 123 fluroescence changes 60 min after toxin exposure. An increase in dihydorhodamine fluorescence indicates increased production of reactive oxygen species. \*\*  $p < .01$ , \*  $p < .05$ ,  $n = 5$ .

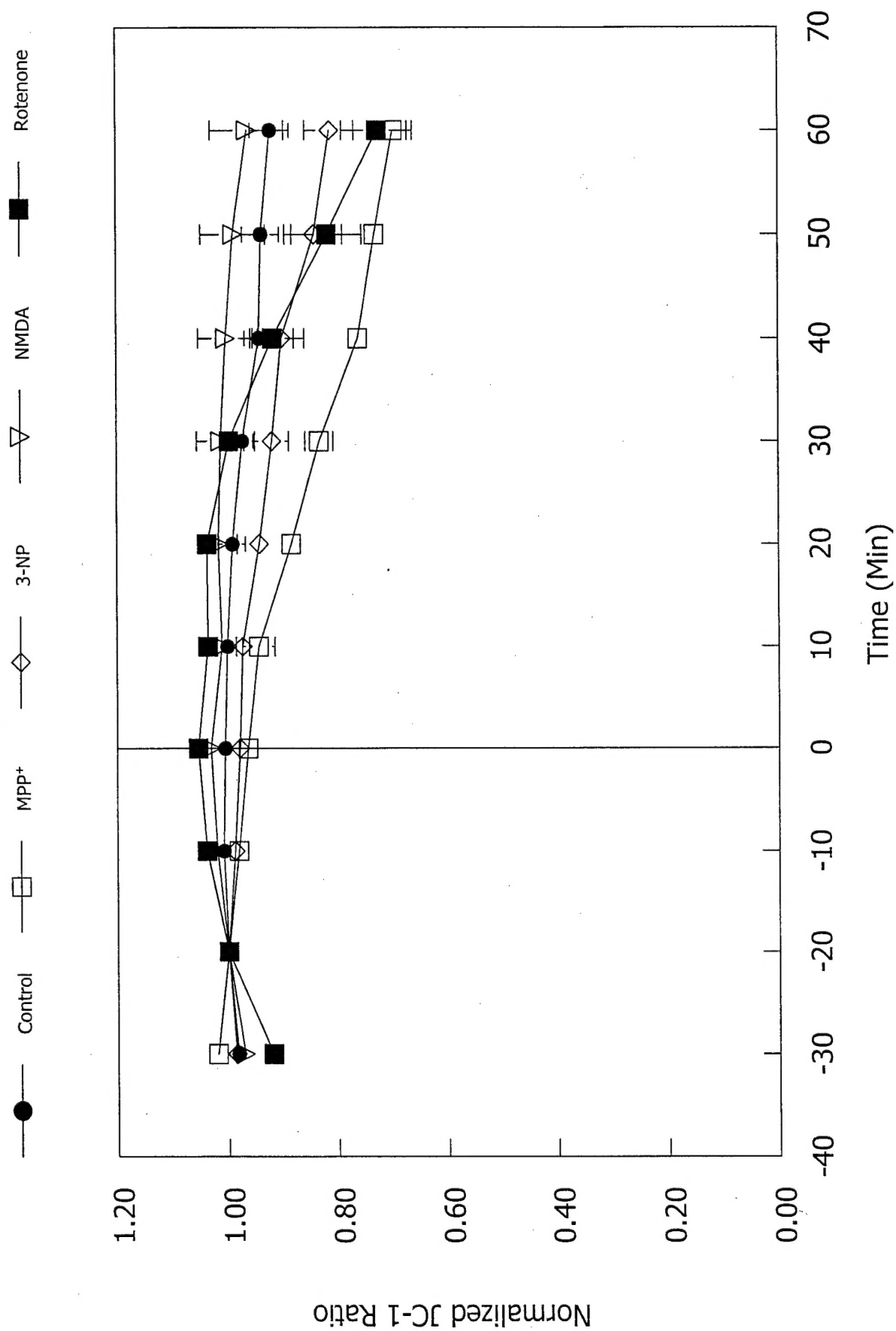


Figure 1

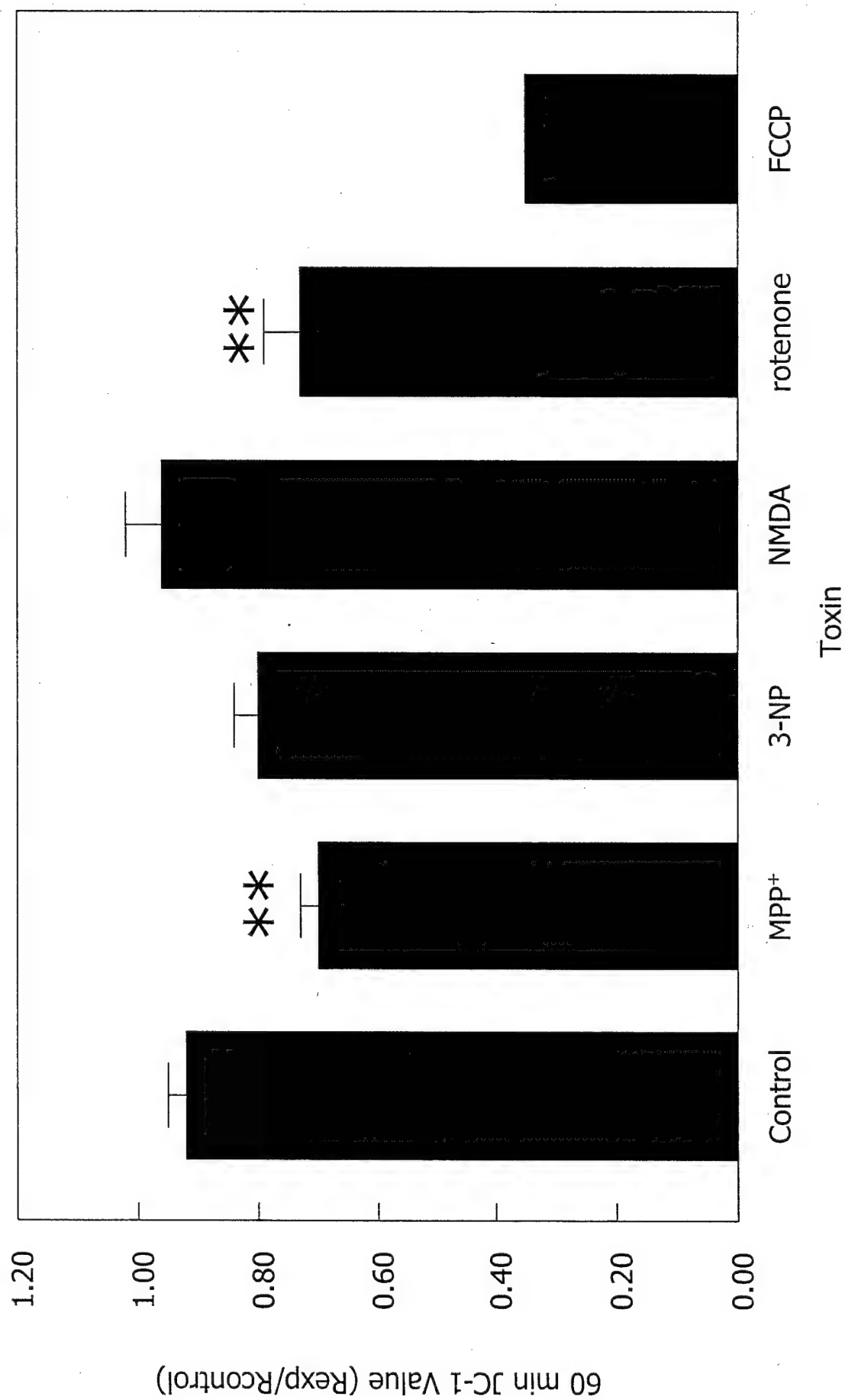


Figure 2

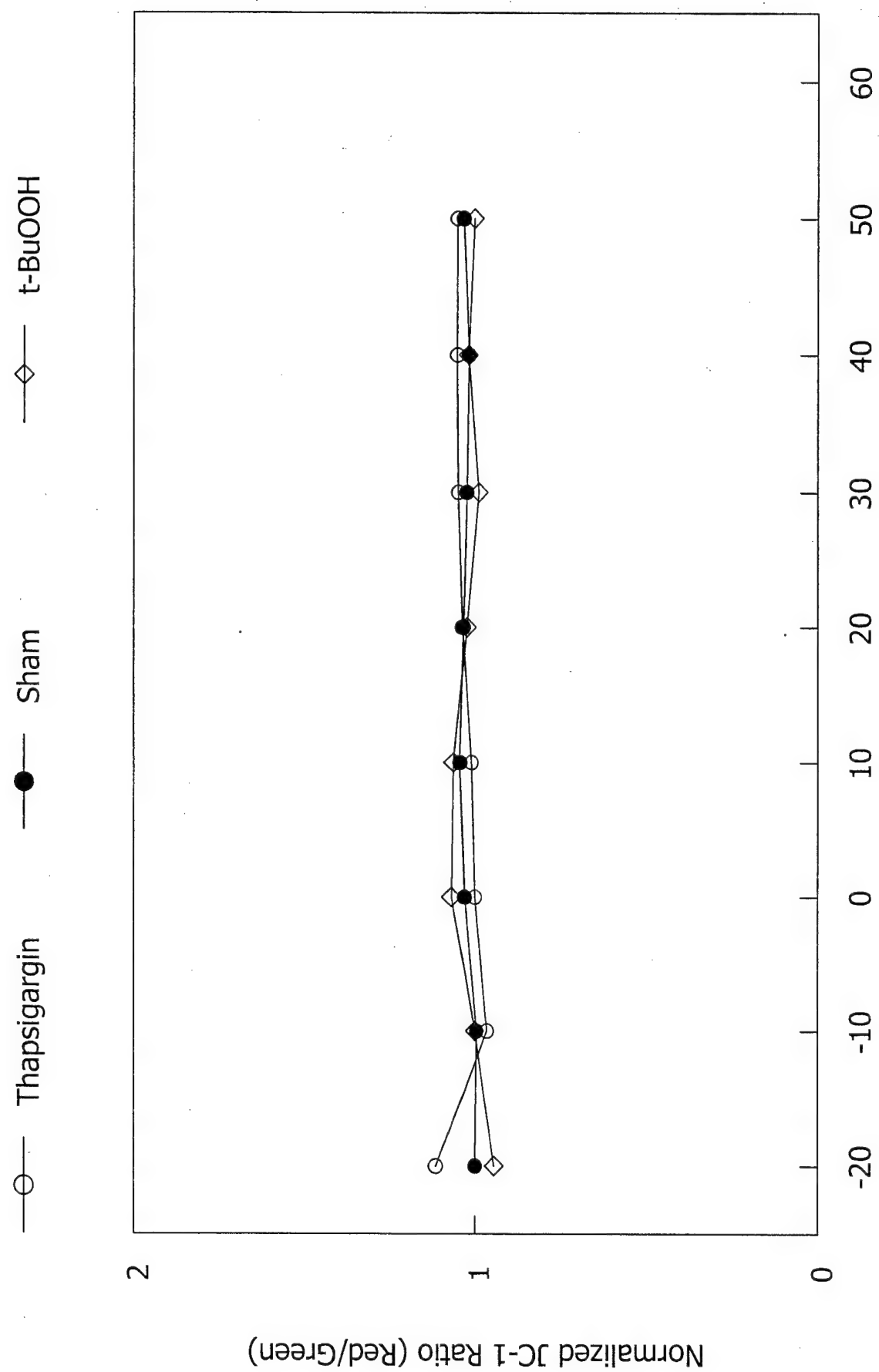


Figure 3

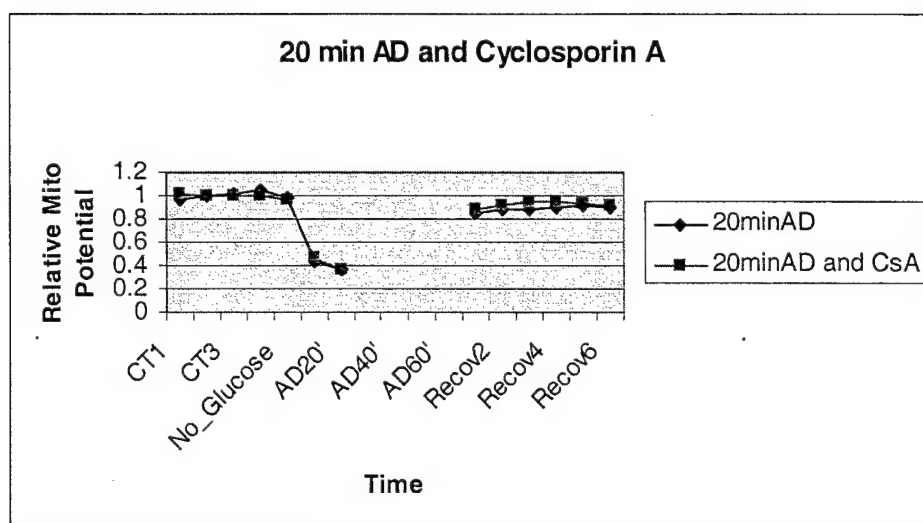
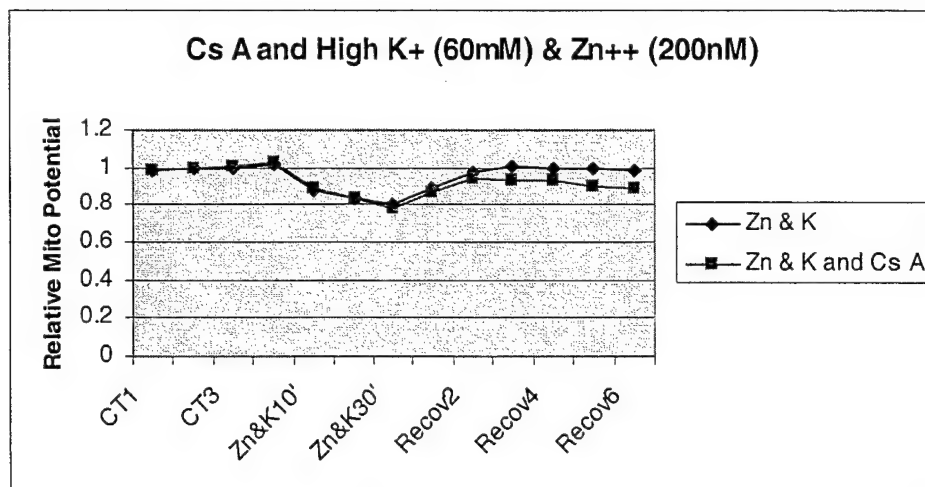


Figure 4

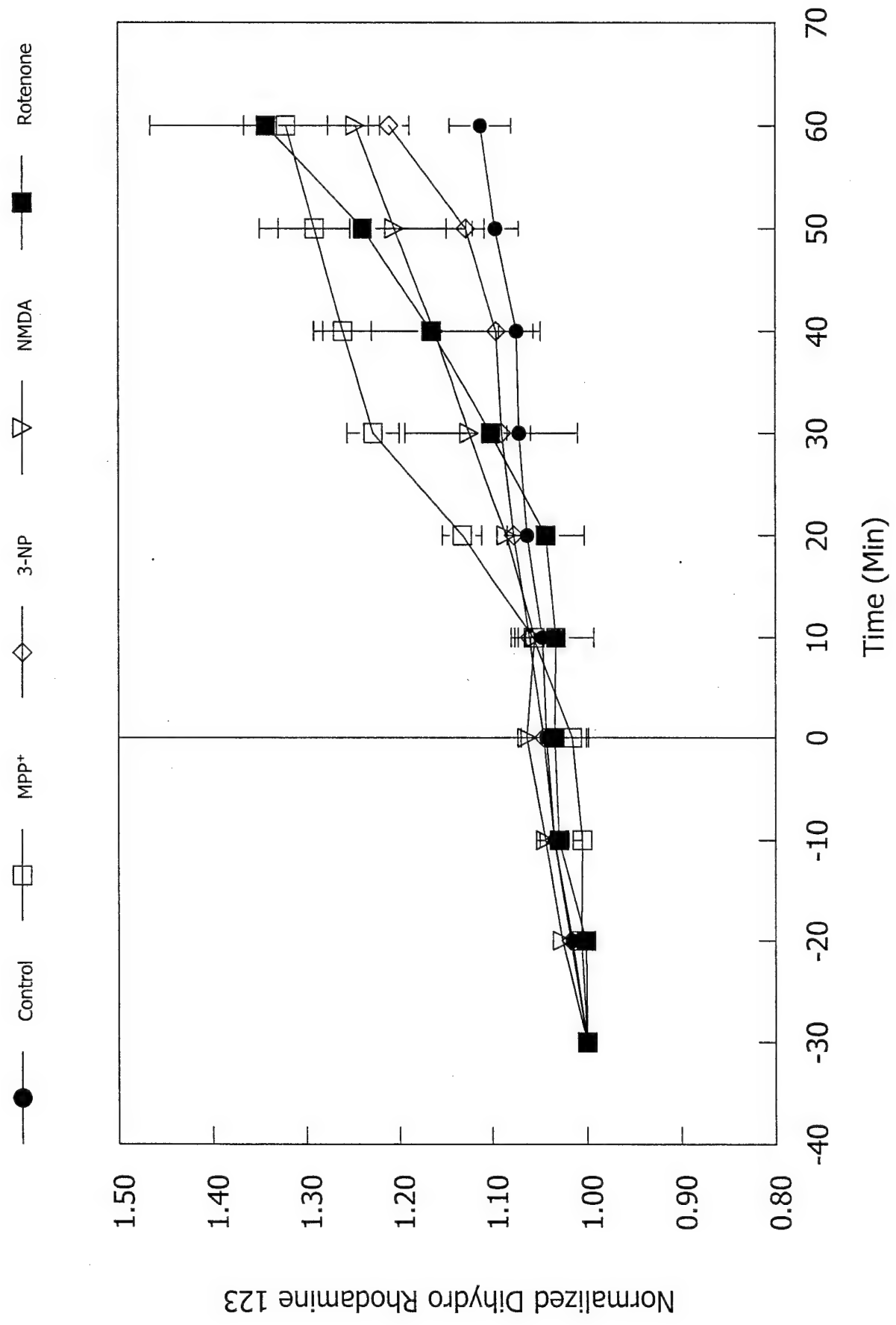


Figure 5

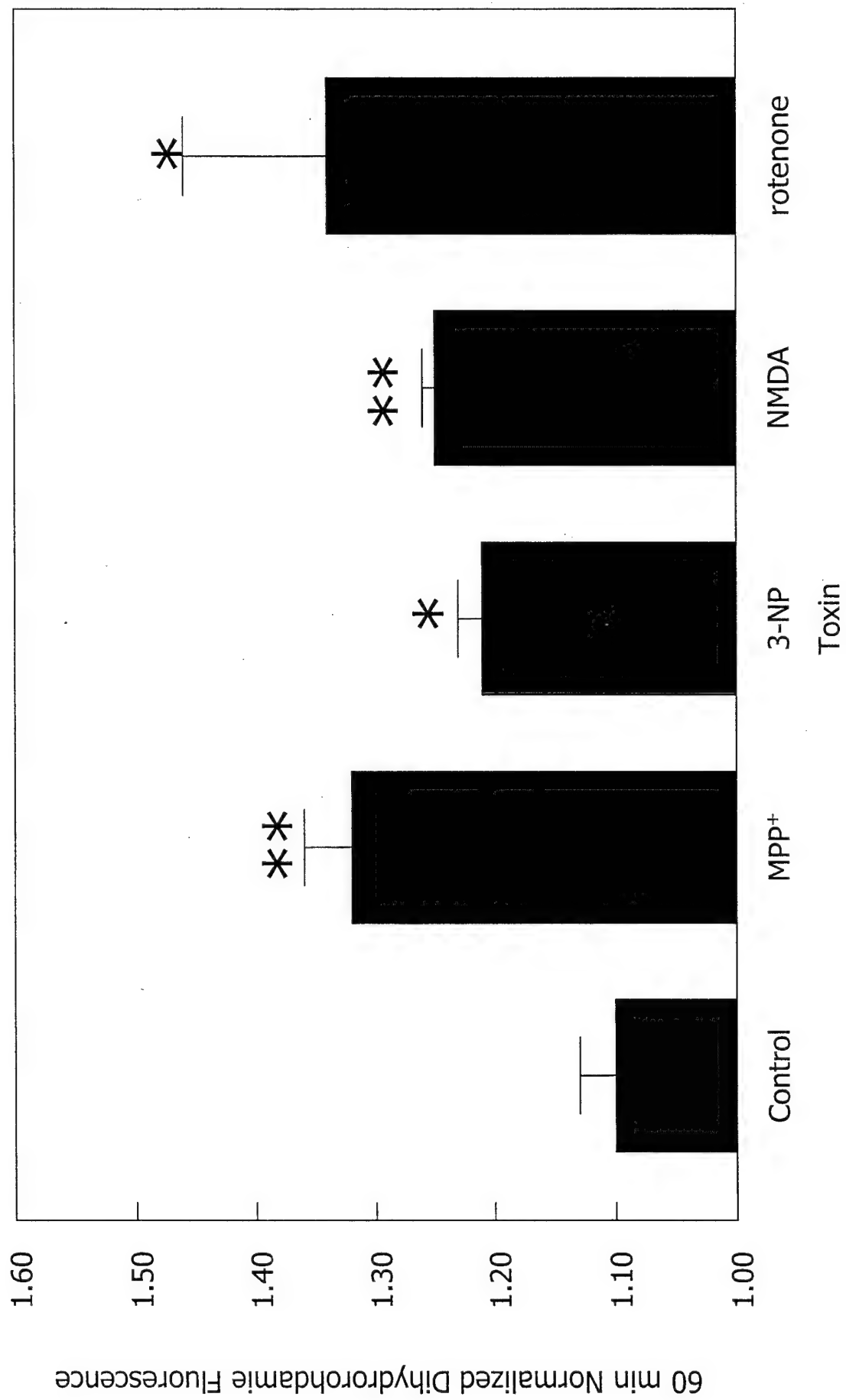


Figure 6

Mitochondrial Complex I Inhibition Produces Selective Damage to Hippocampal  
Subfield CA1 in Organotypic Slice Cultures

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## ABSTRACT

The effects of mitochondrial respiratory chain inhibitors and the excitotoxin N-methyl-D-aspartate (NMDA) on cell death in hippocampal subfields CA1 and CA3 were examined in hippocampal organotypic slice cultures. 2-3 week old slice cultures were exposed for one hour to either the Complex I inhibitors, rotenone or 1-methyl-4-phenylpyridium (MPP<sup>+</sup>), the Complex II inhibitor 3-nitropropionic acid (3-NP), or the excitotoxin NMDA. Cell death was examined 1, 2, or 3 days following treatment by measuring propidium iodide (PI) fluorescence. Rotenone, and to a lesser extent MPP<sup>+</sup> caused greater cell death in hippocampal subfield CA1 than CA3. NMDA caused similar damage in both hippocampal subfields while 3-NP produced little damage in either subfield. The data suggest that mitochondrial Complex I inhibition can produce selective cell damage in hippocampus and in this regard is similar to that observed following hypoxia/ischemia.

Key Words: selective neuronal vulnerability, mitochondrial toxins, excitotoxicity.

## INTRODUCTION

Many neurological disorders are characterized by degeneration of specific neuronal populations. Global ischemia results in selective damage to hippocampal pyramidal cells in the CA1 subfield, dorsolateral striatum, and cortex (Kirino, 1982; Pulsinelli, 1982; Kirino, 2000). Parkinson's disease is characterized by selective damage to dopaminergic neurons in the substantia nigra (Betarbet, 2002). Similarly, selective neuronal degeneration of neurons in nucleus basalis of Maynert and cortical motor neurons are common features of Alzheimer's disease (Whitehouse et al., 1981) and amyotrophic lateral sclerosis (Eisen and Weber, 2001) respectively.

Mitochondria have been implicated as mediators of neuronal death in a variety of neurological conditions (Greenamyre et al., 2001; Beal, 2001; Fiskum, 2000). Patients with Parkinson's disease showed deficiencies of respiratory chain Complex I (Schapira, 1989) and systemic administration of Complex I inhibitors or their precursors produced degeneration of dopaminergic neurons in the substantia nigra in animals (Betarbet, 2002) and man (Langston et al., 1999). Selective damage occurs to dopaminergic neurons in the substantia nigra following systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) occurs because MPTP is oxidized to the toxic metabolite  $MPP^+$  (Langston et al., 1984) which is transported into dopaminergic neurons (Javitch et al., 1985). Toxicity of  $MPP^+$  appears to be, at least in part, due to its ability to inhibit Complex I (Nicklas et al., 1987). Another Complex I inhibitor, rotenone, selectively damages dopaminergic neurons in the substantia nigra although it apparently inhibits Complex I uniformly throughout the brain (Betarbet et al., 2000).

In the present study, we examined the hypothesis that Complex I inhibition would selectively damage non-dopaminergic neurons previously shown to be vulnerable to mitochondrial inhibition from hypoxia/ischemia. We demonstrate here that exposure of hippocampal organotypic slice cultures to Complex I inhibitors results in selective damage to CA1 pyramidal cells similar to that reported following hypoxia/ischemia.

## **MATERIALS AND METHODS**

### **Preparation of Cultures**

Organotypic slice cultures of the hippocampus were prepared according to the methods described by Bergold and Casaccia-Bonnel (1997) and the protocol was approved by the University of Miami, Animal Care and Use Committee. Neonatal Sprague Dawley rats (9-11 days old) were anesthetized by single intraperitoneal injections of ketamine (1.0 mg/pup). The pups were decapitated and the hippocampi dissected free from the cerebral hemispheres and transversely sliced (400  $\mu$ m) on a McIlwain tissue chopper. Slices were incubated in Gey's Balanced Salt Solution (Gibco/Life Technologies) supplemented with 6.5 mg/ml glucose (Sigma Chemical) for 1 hour at 4°C. Slices were then transferred onto 30-mm diameter membrane inserts (Millicell-CM, Millipore), and were transferred to 6 well culture trays with 1 ml of slice culture medium per well. The slice culture medium consisted of 50 % Minimum Essential Medium (Gibco/Life Technologies), 25 % Hank's balanced salt solution

(Gibco/Life Technologies), 25 % heat inactivated horse serum (Gibco/Life Technologies) supplemented with 6.5 mg/ml glucose and glutamine. The cultures were maintained at 37 °C in an incubator (Nuair, CF autoflow) with an atmosphere of humidified 21% O<sub>2</sub> - 5% CO<sub>2</sub>. The slice culture medium was changed twice a week and slices were cultured for 14-15 days before experiments.

### **Assessment of Cell Death**

The number of dead or dying cells in hippocampal subfields CA1 and CA3 was estimated by fluorescent staining with Propidium Iodide (PI, from Sigma). Prior to experimental treatment (exposure to mitochondrial inhibitors), slices were incubated in culture medium supplemented with 2 µg/ml PI for 1 hr. Images of PI fluorescence in organotypic slices were acquired using an inverted fluorescence microscope (Olympus IX 50) and Spot CCD camera (Diagnostic Instruments Inc., Sterling Heights MI) and SPOT advanced software. Images of cultured slices were taken prior to toxin exposure, to estimate background fluorescence, and again 24 hr, 48 hr, and 72 hr later. Finally, the slices were superfused with NMDA (100 µM)(from Sigma) for 1 hr and a terminal image was acquired 24 hr later to estimate maximum cell death. For quantitation, regions of interest (ROIs) corresponding to hippocampal subfields CA1 and CA3 were selected from bright field images of each slice using Scion Image software (Windows Version). ROIs were transferred to individual images in an image stack after optical alignment of brightfield images. Relative cell death was calculated from each ROI as follows:

Relative % cell death =  $(F_{exp} - F_{min}) / (F_{max} - F_{min}) * 100$ , where  $F_{exp}$  is the fluorescence of the test condition,  $F_{max}$  is maximum fluorescence observed during an

experiment (following toxin exposure or terminal treatment with 100  $\mu$ M NMDA for 1 hr), and Fmin is background fluorescence (prior to treatment).

### **Mitochondrial Inhibitors**

Hippocampal organotypic slice cultures were treated for 1 hr with either the Complex I inhibitors rotenone (0.1  $\mu$ M, 1  $\mu$ M, 10  $\mu$ M) or 1-methy-4-phenylpyridinium (MPP<sup>+</sup>, 10  $\mu$ M, 100 $\mu$ M, 1 mM), the Complex II inhibitor 3-nitropropionic acid (10  $\mu$ M, 100 $\mu$ M, 1 mM), or the excitotoxin NMDA (1  $\mu$ M, 10  $\mu$ M, 100 $\mu$ M). All compounds were dissolved in culture medium (see above) except rotenone which was dissolved in culture medium containing 0.1% ethanol. Sham treated slices were incubated for 1 hr in exchanged culture medium or culture medium containing 0.1% ethanol (vehicle for rotenone).

### **Statistics**

Experimental groups were compared statistically using Analysis of Variance (ANOVA).

## **RESULTS**

Hippocampal organotypic slice cultures were a reliable model for assessing cell death following exposure to mitochondrial toxins. Little background PI fluorescence was observed in hippocampal subfields CA1 And CA3 prior to toxin exposure or in sham-treated slices, indicating limited cell death in untreated slices after 2-3 weeks of culture. However, many slices showed some damaged cells in the upper blade of the dentate

gyrus prior to toxin exposure. The variability in the dentate gyrus precluded any meaningful assessment of the effects of toxin exposure in this hippocampal subfield.

### **Rotenone**

Hippocampal slices were exposed for 1 hr either to vehicle, 0.1  $\mu$ M, 1.0  $\mu$ M, or 10  $\mu$ M concentrations of the mitochondrial Complex I inhibitor, rotenone. Examples of images showing the changes in PI fluorescence taken prior to exposure, and each of 3 days after exposure, are shown in figure 1. Quantitative assessment of cell death in hippocampal subfields CA1 and CA3 following rotenone exposure are shown in Figure 2. Rotenone treatment resulted in a highly significant dose-dependent increase in cell death in hippocampal pyramidal cells both in CA1 and CA3 (Main effect of dose,  $F = 84.5$ ,  $df\ 3,24$ ,  $p < .001$ ,  $n = 4$ ). Significantly, cell death following rotenone exposure was more pronounced at lower doses in subfield CA1 than in subfield CA3 (Region X Dose interaction,  $F = 5.4$ ,  $df\ 3,24$ ,  $p < .01$ ,  $n = 4$ ). For example, PI fluorescence following exposure to 1  $\mu$ M rotenone was  $30.1 \pm 11.1\%$ ,  $77.1 \pm 10.9\%$  and  $85.1 \pm 16.5\%$  on days 1, 2, and 3 respectively in CA1, and  $9.4 \pm 5.5\%$ ,  $20.3 \pm 10.7\%$ , and  $58.5 \pm 20.5\%$  on days 1, 2, and 3 in CA3. Higher doses of rotenone (10  $\mu$ M) resulted in significant cell death in both hippocampal subfields.

### **1-Methyl-4-Phenylpyridium (MPP<sup>+</sup>)**

Examples of PI fluorescence images of slice cultures exposed to 10  $\mu$ M, 100  $\mu$ M, or 1 mM of the respiratory chain Complex I inhibitor MPP<sup>+</sup>, are shown in figure 3. Quantitative analysis of PI fluorescence following MPP<sup>+</sup> exposure is shown in figure 4. Similar to rotenone, MPP<sup>+</sup> caused dose-dependent death of pyramidal cells in both CA1 and CA3 (Main Effect of Dose,  $F = 10.2$ ,  $df\ 3,24$ ,  $p < 0.001$ ,  $n = 4$ ). After exposure to 1

mM MPP<sup>+</sup>, cell death as indicated by PI fluorescence was  $42.8 \pm 17.1 \%$ ,  $75.5 \pm 23.8 \%$ , and  $73.3 \pm 20.6 \%$  on days 1, 2, and 3 respectively in subfield CA1. In subfield CA3, PI fluorescence was  $25.0 \pm 14.5 \%$ ,  $44.7 \pm 26.4 \%$ , and  $42.3 \pm 17.8 \%$  on days 1, 2, and 3 after exposure to 1 mM MPP<sup>+</sup>. However, while there was a trend for damage to be greater in CA1 than CA3 following MPP<sup>+</sup> exposure, the difference did not reach statistical significance (Dose X Region interaction,  $F = 1.3$ ,  $df\ 3,24$ ,  $p = 0.3$ ,  $n = 4$ ).

### **3-Nitropropionic Acid (3-NP)**

In comparison to the Complex I inhibitors rotenone and MPP<sup>+</sup>, the Complex II inhibitor 3-NP had a much smaller effect on pyramidal cell death (Main Effect of Dose,  $F = 3.1$ ,  $df\ 3,24$ ,  $p < .05$ ,  $n = 4$ ). Also, there was no tendency for 3-NP to selectively damage either subfield (Dose X Region interaction,  $F = 0.6$ ,  $df\ 3,24$ ,  $p = 0.6$ ,  $n = 4$ ). A summary of the data is presented in figure 5 (Images not shown).

### **N-Methyl-D-Aspartate (NMDA)**

The excitotoxin NMDA caused pyramidal cell death only at the highest dose (100  $\mu$ M) tested (Main Effect of Dose,  $F = 416$ ,  $df = 3,24$ ,  $p < .0001$ ,  $n = 4$ ). Examples of PI fluorescence images acquired before and after exposure of organotypic slice cultures to 1  $\mu$ M, 20  $\mu$ M, or 100  $\mu$ M NMDA are shown in figure 6. A summary of the data is shown in figure 7. In contrast to rotenone, and tentatively  $MPP^+$ , NMDA did not produce selective damage in subfield CA1 compared to CA3 (Dose X Region interaction  $F = 1.5$ ,  $df = 3,24$ ,  $p = 0.2$ ,  $n = 4$ ).

### **DISCUSSION**

In this report we demonstrate that inhibition of mitochondrial respiratory chain Complex I results in selective damage to pyramidal cells in subfield CA1. Selective neuronal damage was not observed following inhibition of Complex II with 3-NP or following exposure to the excitotoxin NMDA. We believe this is the first report that Complex I inhibition causes selective damage to CA1 pyramidal cells in the hippocampus.

Alterations in Complex I activity of the mitochondrial respiratory chain have typically been associated with Parkinson's disease and damage to dopaminergic neurons in the substantia nigra and striatum. MPTP caused selective damage to neurons in the substantia nigra and striatum in man (Langston et al., 1999), animals (Betarbet, 2002) and produced neurological symptoms similar to those found in Parkinson's disease (Langston et al., 1983). MPTP appears to selectively damage dopaminergic neurons



though its conversion to the Complex I inhibitor MPP<sup>+</sup> (Langston et al., 1984), and transport of MPP<sup>+</sup> selectively into synaptic terminals of dopaminergic neurons (Javitch et al., 1985). It has also been reported that patients with Parkinson's disease may show a deficiency in mitochondrial Complex I ((Schapira, 1989; Parker et al., 1989). More recently, it was reported that chronic systemic administration of rotenone, another Complex I inhibitor, caused selective degeneration of dopaminergic neurons in the striatum and substantia nigra (Betarbet et al., 2000).

In contrast, selective damage to pyramidal cells in hippocampal subfield CA1 has been commonly reported following global ischemia *in vivo* (Kirino, 1982, and Kirino, 2000). Oxygen-glucose deprivation (in vitro ischemia) has also been shown to selectively damage CA1 pyramidal cells in hippocampal organotypic slice cultures (Laake et al., 1999). We report here that selective damage to CA1 pyramidal cells also occurred after treatment of hippocampal organotypic slice cultures with the Complex I inhibitor rotenone. Betarbet et al (2000) did not report damage to CA1 pyramidal cells after systemic rotenone infusion *in vivo*. However it was unclear whether damage did not occur or whether a detailed analysis of hippocampus pathology was not conducted. Also, in their study exposure to rotenone was chronic and at a lower dose than used here in organotypic slice cultures.

The mechanism of rotenone-induced cell damage in hippocampal subfield CA1 is currently unknown. However, because a similar pattern of damage occurs with oxygen-glucose deprivation and rotenone treatment, it is safe to speculate that the mechanism of damage also is similar. One such possibility is the generation of reactive oxygen species (ROS). Oxygen-glucose deprivation results in increase ROS generation in organotypic

hippocampal slice cultures (Frantseva et al., 2001). Complex I inhibition with rotenone also increase ROS production in hippocampal slices (Saybasili et al., 2001). However, if ROS production is key to selective neuronal damage in CA1 pyramidal cells, the increase should be greater in CA1 than in CA3 following either oxygen-glucose deprivation or Complex I inhibition. To date, selective ROS production in CA1 has not been examined.

Elevation of intracellular calcium could also mediate selective neuronal vulnerability of CA1 pyramidal cells following either Complex I inhibition or oxygen-glucose deprivation. Intracellular calcium levels were clearly elevated following oxygen glucose deprivation (Frantseva et al. 2001) and more importantly the increase occurred earliest and was most severe in subfield CA1 (Mitani, 1994). Complex I inhibition also results in elevation of intracellular calcium (Leist et al., 1998) but experiments have not been conducted to compare changes in hippocampal subfields CA1 and CA3.

We did not observe reliable selective hippocampal CA1 pyramidal cell death following treatment either with the excitotoxin, NMDA, or the mitochondrial Complex II inhibitor 3-NP. Previous studies (Kristensen et al., 2001; Prendergast et al., 2001) also showed comparable cell death in subfields CA1 and CA3 following exposure to high (>100  $\mu$ M) concentrations of NMDA. However, Kristensen et al (2001) reported selective damage to CA1 pyramidal cells after 2 days of exposure to 10  $\mu$ M NMDA. There have been no previous studies examining selective neuronal vulnerability in hippocampus following inhibition of mitochondrial Complex II. In fact, treatment with 3-NP has been shown to “pre-condition” or protect hippocampal neurons from subsequent hypoxia/ischemia (Sugino et al. 1999).

In conclusion, we have demonstrated selective damage to pyramidal cells in subfield CA1 of hippocampal organotypic slice cultures following inhibition of Complex I of the mitochondrial respiratory chain. Selective damage to neurons in subfield CA1 was similar to that observed by other investigators after hypoxia/ischemia. Thus, it appears that severe inhibition of respiratory chain function may be sufficient to trigger CA1 neuronal death, whether inhibition occurs as a result of ischemia or exposure to mitochondrial toxins.

#### *acknowledgements*

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## FIGURE LEGENDS

Figure 1. Examples of brightfield and propidium iodide (PI) fluorescence images of hippocampal organotypic slice cultures. Rotenone (10  $\mu$ M, 1  $\mu$ M, or 0.1  $\mu$ M) was applied for 1 hr after acquiring background images on Day 0. Final PI fluorescence was obtained 24 hr after applying 100  $\mu$ M NMDA for 1 hr on Day 3.

Figure 2. Average values (Mean  $\pm$  SEM) of propidium iodide (PI) fluorescence in hippocampal subfields CA1 and CA3 on Days 1, 2, and 3 after exposure to either vehicle (0.1% ethanol), 0.1  $\mu$ M, 1.0  $\mu$ M, or 10  $\mu$ M rotenone. Statistical comparisons of groups are provided in the text.

Figure 3. Examples of brightfield and propidium iodide (PI) fluorescence images of hippocampal organotypic slice cultures. 1-methy-4-phenylpyridium (MPP<sup>+</sup>, 1 mM, 100  $\mu$ M, or 10  $\mu$ M) was applied for 1 hr after acquiring background images on Day 0. Final PI fluorescence was obtained 24 hr after applying 100  $\mu$ M NMDA for 1 hr on Day 3.

Figure 4. Average values (Mean  $\pm$  SEM) of propidium iodide (PI) fluorescence in hippocampal subfields CA1 and CA3 on Days 1, 2, and 3 after exposure to 1 mM, 100  $\mu$ M, or 10  $\mu$ M MPP<sup>+</sup>. Statistical comparisons of groups are provided in the text.

Figure 5. Examples of brightfield and propidium iodide (PI) fluorescence images of hippocampal organotypic slice cultures. N-methyl-D-aspartate (NMDA, 100  $\mu$ M, 10  $\mu$ M or 1  $\mu$ M) was applied for 1 hr after acquiring background images on Day 0. Final PI fluorescence was obtained 24 hr after applying 100  $\mu$ M NMDA for 1 hr on Day 3.

Figure 6. Average values (Mean  $\pm$  SEM) of propidium iodide (PI) fluorescence in hippocampal subfields CA1 and CA3 on Days 1, 2, and 3 after exposure to 100  $\mu$ M, 10  $\mu$ M, or 1  $\mu$ M NMDA. Statistical comparisons of groups are provided in the text.

Figure 7. Average values (Mean  $\pm$  SEM) of propidium iodide (PI) fluorescence in hippocampal subfields CA1 and CA3 on Days 1, 2, and 3 after exposure to 1 mM, 100  $\mu$ M, or 10  $\mu$ M, 3-nitropropionic acid (3-NP). Statistical comparisons of groups are provided in the text.

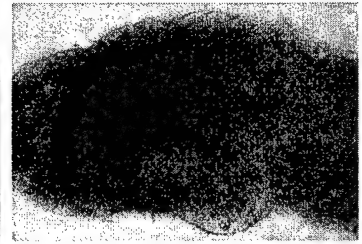
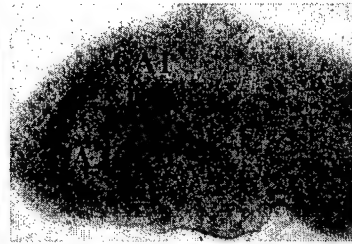
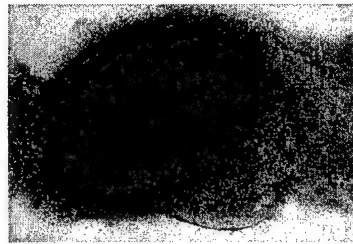
# Rotenone

10  $\mu$ M

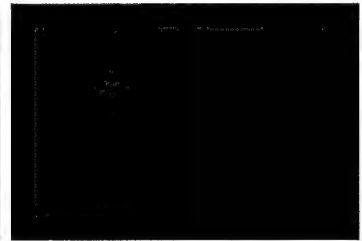
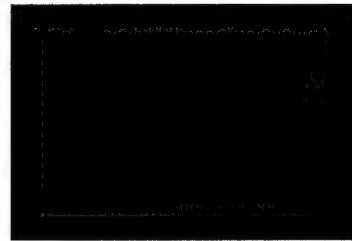
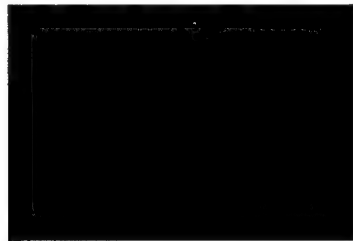
1  $\mu$ M

0.1  $\mu$ M

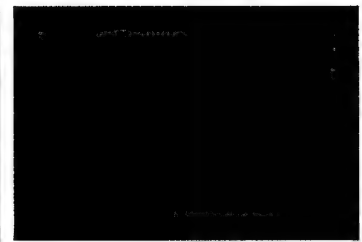
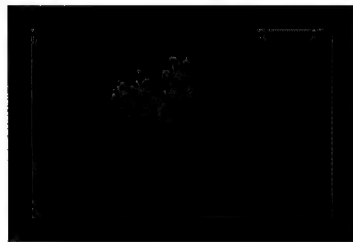
Brightfield



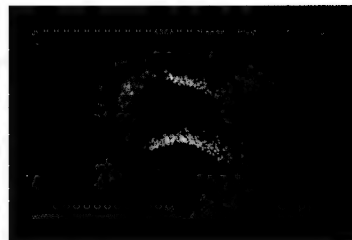
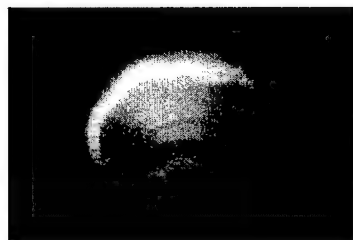
Day 0



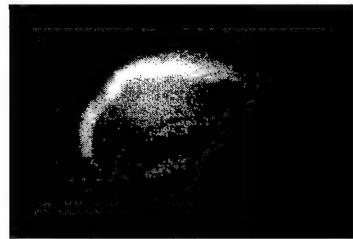
Day 1



Day 2



Day 3



Final

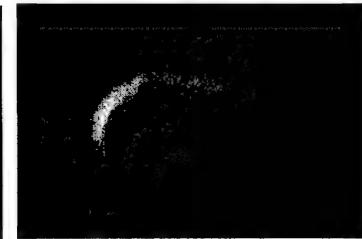
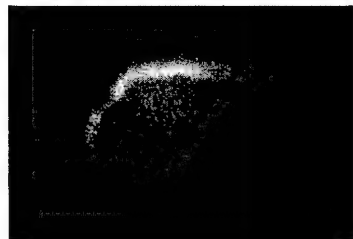


Figure 1

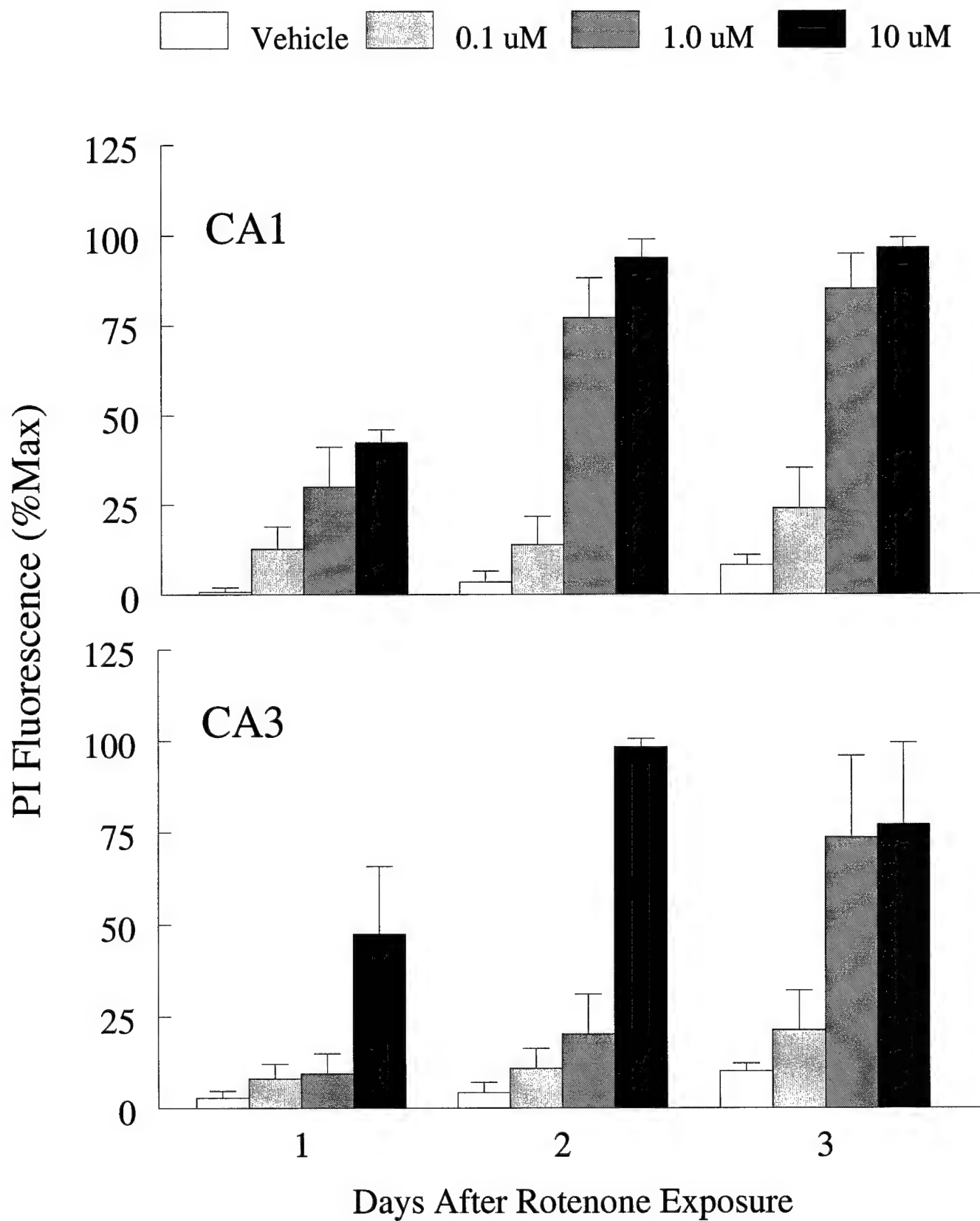


Figure 2



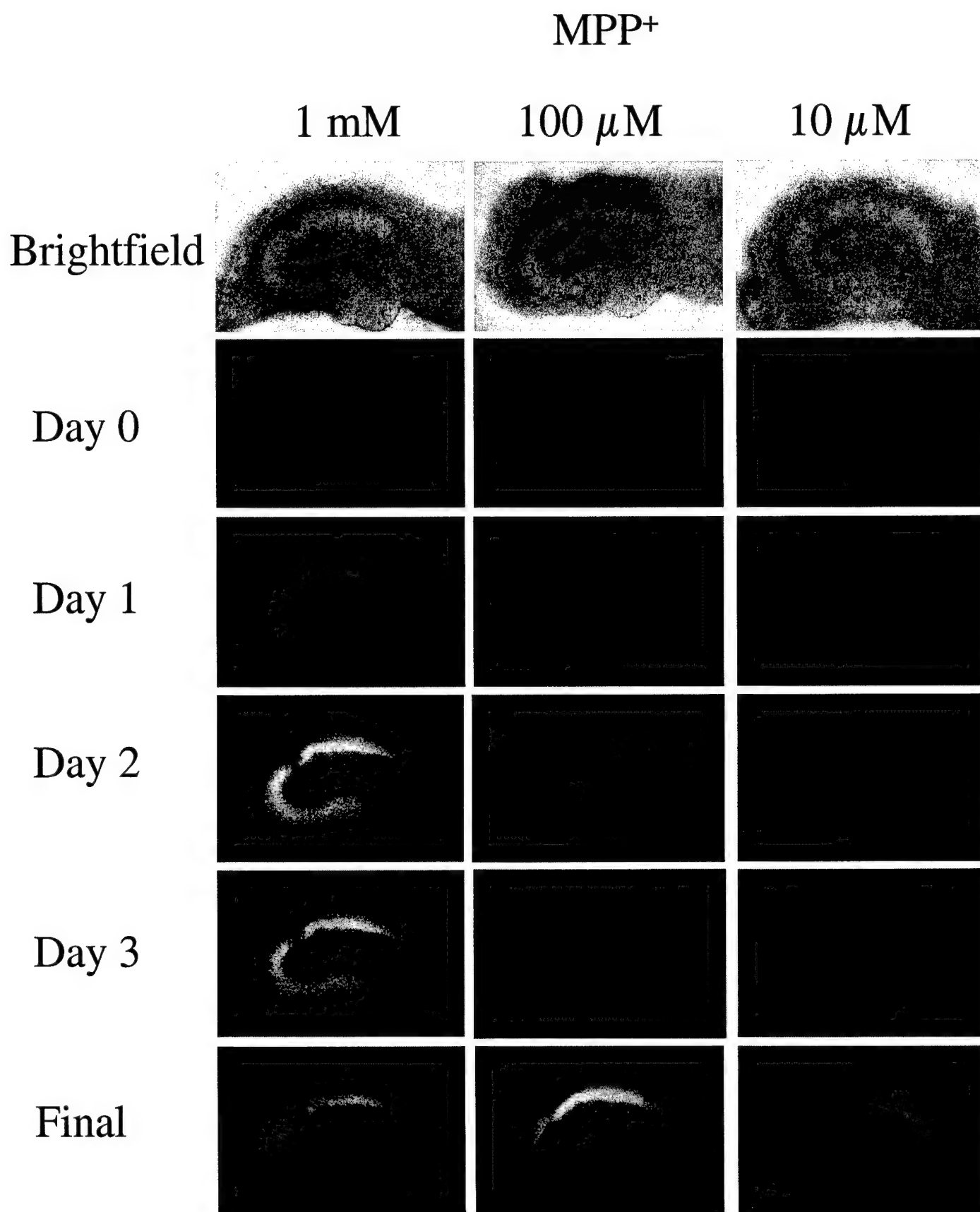


Figure 3

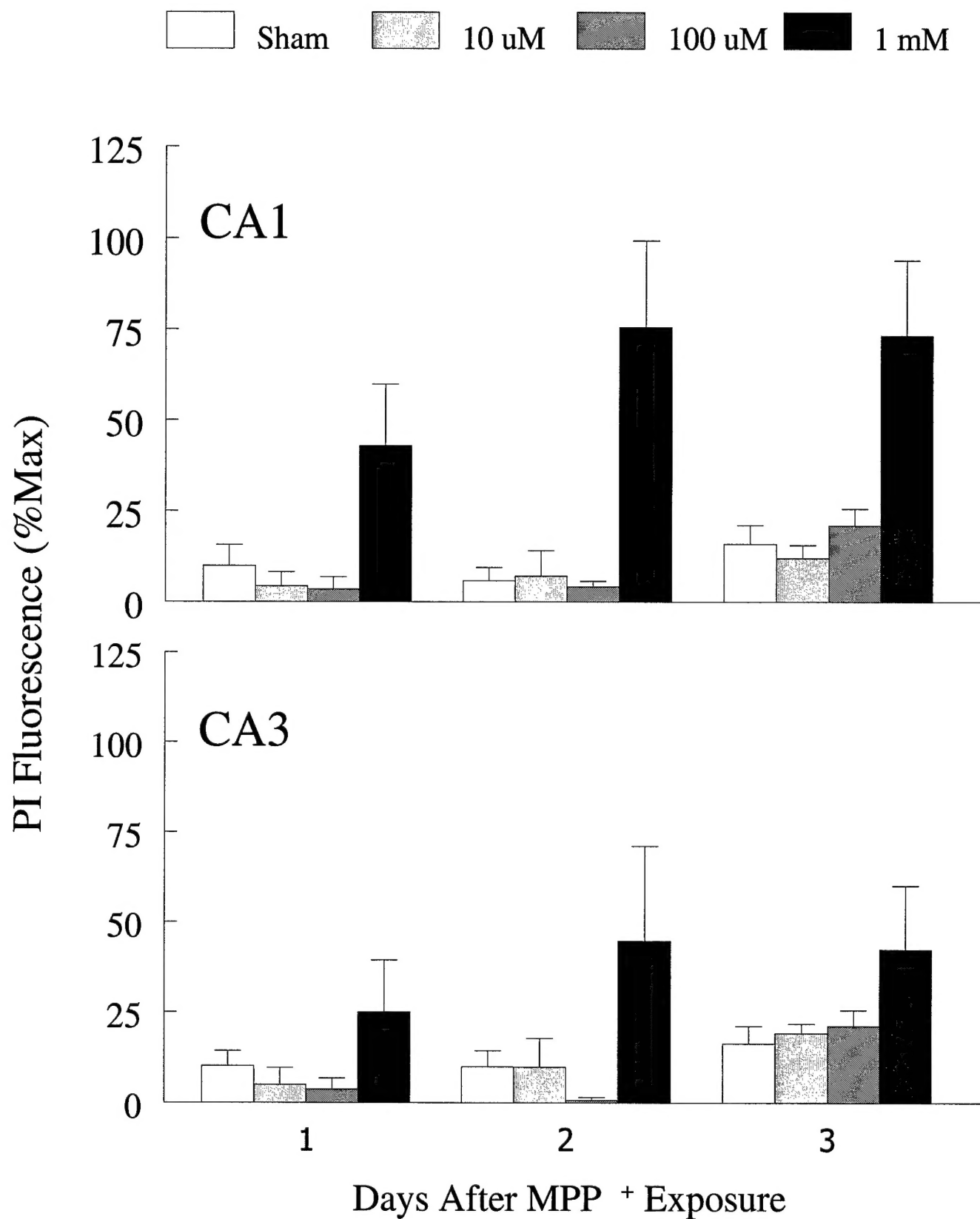


Figure 4

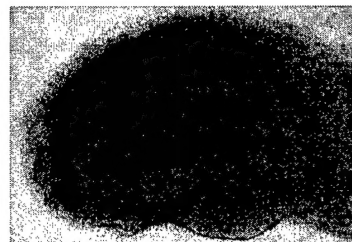
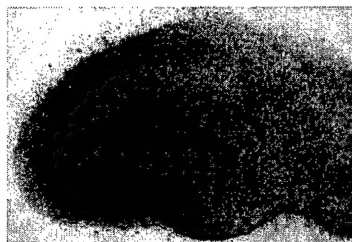
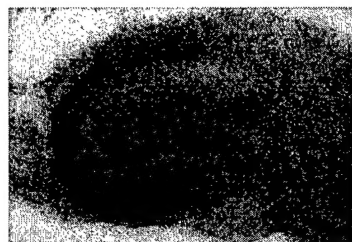
# NMDA

100  $\mu\text{M}$

10  $\mu\text{M}$

1  $\mu\text{M}$

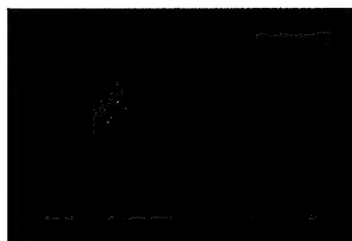
Brightfield



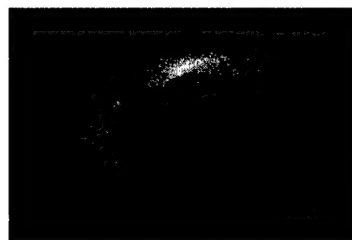
Day 0



Day 1



Day 2



Day 3



Final



Figure 5

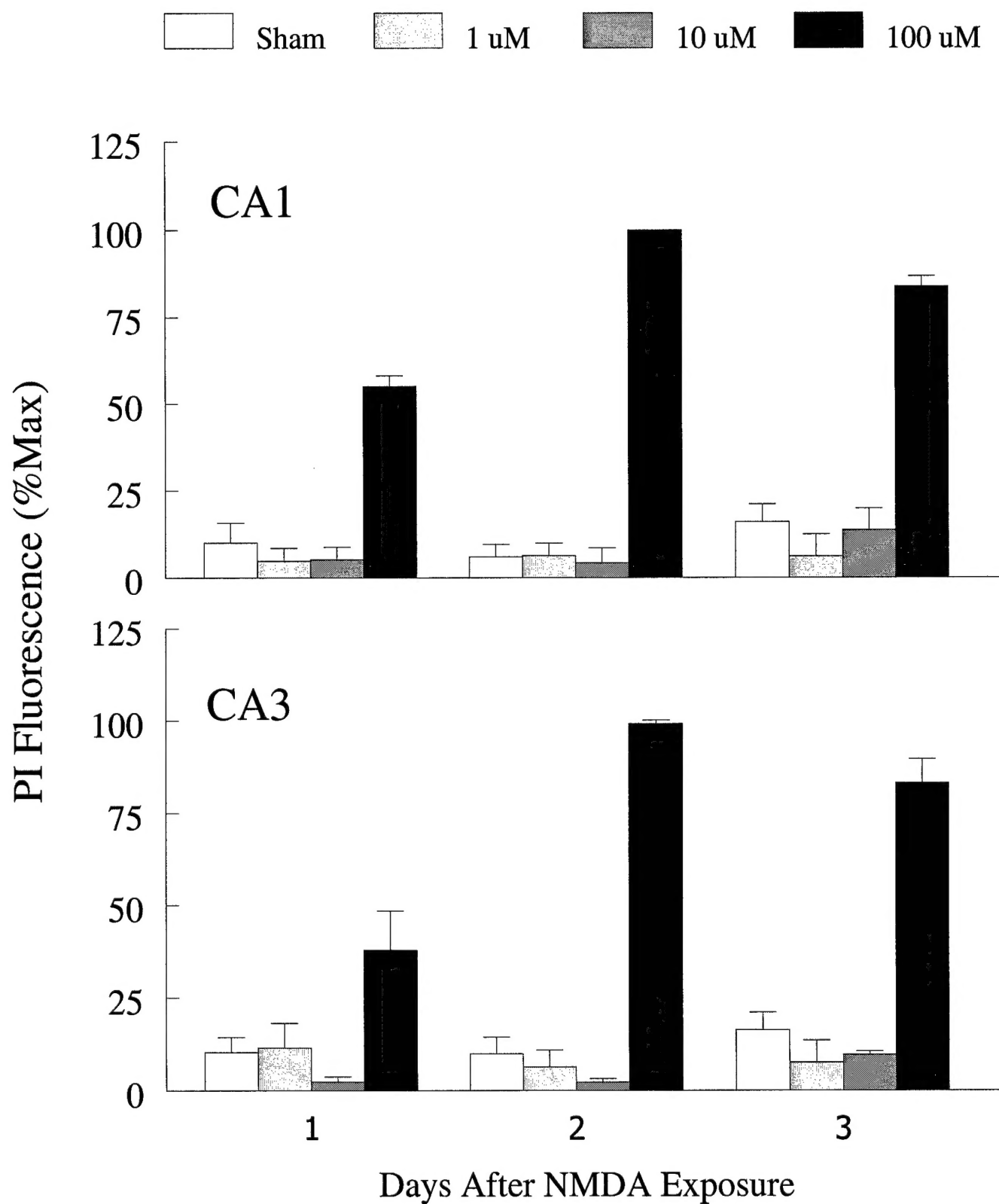


Figure 6

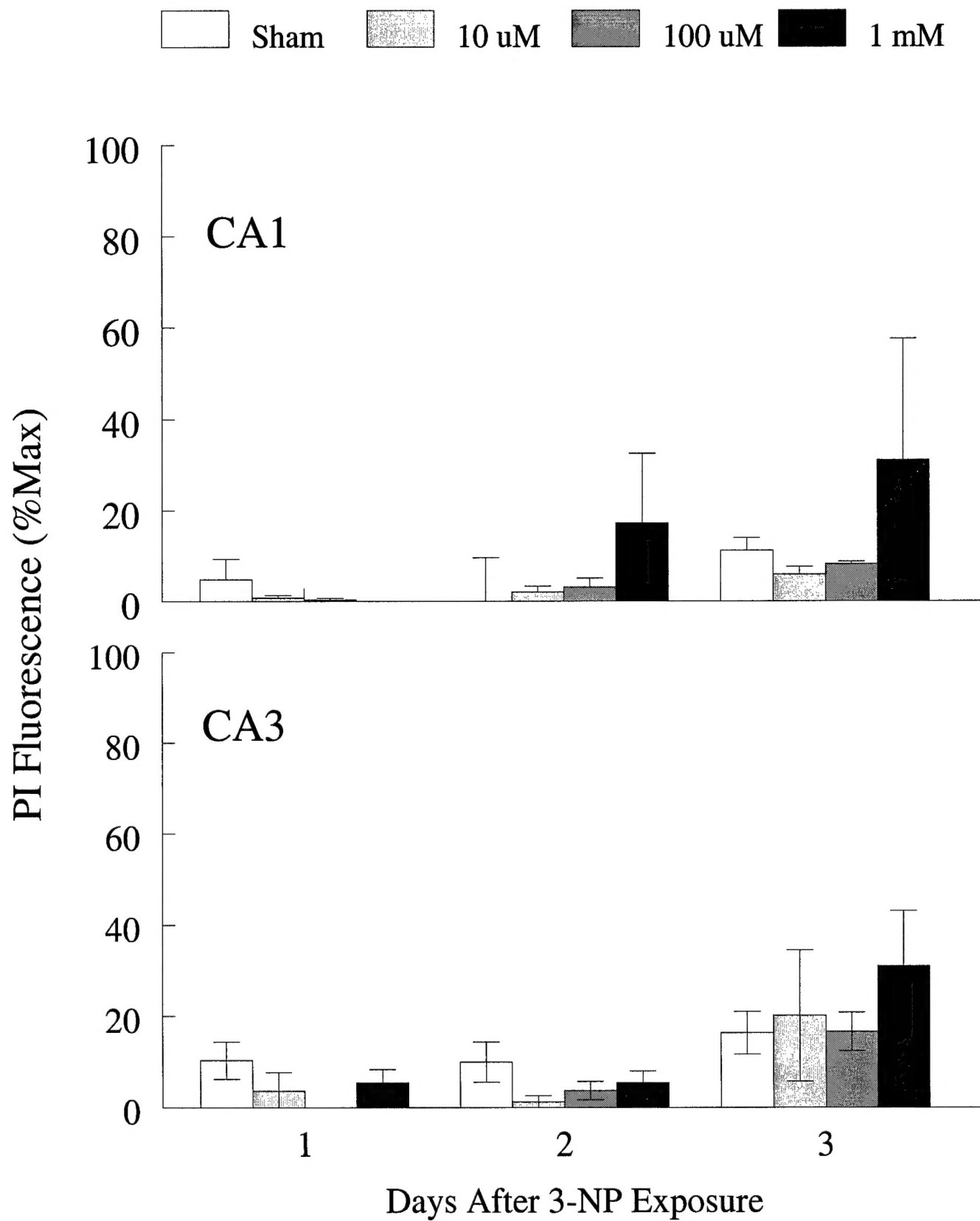


Figure 7